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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
While the relevance of estrogen to established breast cancer is well documented, the role of estrogen in breast cancer initiation is still unclear. The carcinogenic effect of estrogen is mediated by its genotoxic metabolites. We hypothesized that increases in estradiol concentration in breast tissue will lead to accumulation of genotoxic metabolites and eventually causes breast cancer. To test this hypothesis, we proposed to overexpress aromatase into a benign breast epithelial cell line, MCF-10A and to determine the production of genotoxic metabolites of estrogen and cell transformation. During the first year of funding, we successfully constructed pTRE-arom vector. Using our MCF-7Tet-off cells, we demonstrated that this vector expresses functional aromatase which is tightly controlled by tetracycline. Meanwhile, we attempted to establish MCF-10ATet-off cell line that is required for tetracycline-controlled expression of aromatase. However, MCF-10A seems not suitable for tetracycline-controlled gene expression because it showed very high basal expression of the gene tested (luciferase reporter gene) and tetracycline does not regulate gene expression. Therefore, we used alternative approach to establish a stable line of MCF-10A that expresses high levels of aromatase. We fulfilled the tasks scheduled for the first year of funding. The resultant MCF-10A^{arom} cells are ready for the future studies.

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INTRODUCTION

The relevance of estrogen to established breast cancer is well documented. Mediated by the estrogen receptor, estrogen stimulates cell proliferation and promotes the progression of hormone-dependent breast cancer. The fact that women with bilateral oophorectomy before the age 35 had a substantial lower lifetime risk of developing breast cancer (1) suggests that estrogen also play a role in breast cancer initiation. Data from the studies using animal models where estrogen induced tumors indicate that estrogen can be metabolized to hydroxylated catechol estrogen and cause genotoxicity (2-4). This mechanism together with receptor-mediated stimulation of cell proliferation may act in concert to induce breast cancer. Based on the data from clinical studies and our animal studies, in situ aromatization in the breast makes the major contribution to the high tissue estrogen concentrations (5,6). We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test this hypothesis, we proposed to establish a model of aromatase overexpression using a benign breast epithelial cell line, MCF-10A and to determine whether overexpression of aromatase will lead to accumulation of genotoxic metabolites of estrogen and cell transformation.

BODY

The first step of the proposal is to establish the model system (Specific Aim 1). The goal of this step is to obtain a benign breast epithelial cell line which stably expresses inducible human aromatase cDNA. The tasks involved are: 1) construction of the expression vector for aromatase cDNA; 2) stable transfection of MCF-10A cells with pTet-Off vector; 3) second stable transfection of MCF-10A cells with pTRE-arom, the aromatase cDNA under the control of tetracycline response element (TRE); and 4) characterization of aromatase-transfected MCF-10A cells.

I. Construction of pTRE-arom vector.

CLONTECH Tet-Off gene expression system was employed. PTRE2 vector (Fig. 1) is the response plasmid for expression of aromatase under the control of TRE. The restriction enzymatic sites *Mlu I* and *Xba I* were selected for insertion of aromatase cDNA. A pair of primers were designed to include the sequence recognized by *Mlu I* upstream the aromatase cDNA and the sequence recognized by *Xba I* downstream the aromatase cDNA. The sequences of the primers are as follows: 5'-CGACGCGTGCCACCATGGTTTTGGAAATG C-3' (forward primer) and 5'-GCTCTAGACTCTAGTGTTCCAGACA-3' (reverse primer). Using pHβ-aro plasmid as a template, aromatase cDNA with appropriate subcloning sequences was amplified by PCR. The PCR product was verified by sequencing analysis. The resultant PCR product (1.5 kb) and pTRE-2 vector were digested with *Mlu I* and *Xba I*, column purified, and ligated. DH5α cells were transformed with pTRE-arom construct. Transformed DH5α cells were grown in the presence of ampicillin. Six colonies were picked up, expanded, and plasmid DNA prepared. One clone carried the aromatase cDNA. Fig. 2 shows restriction enzyme analysis of the plasmid from this clone.

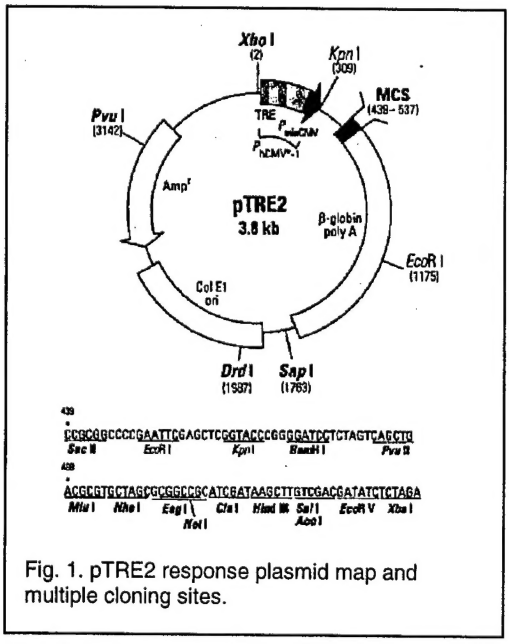

Fig. 1. pTRE2 response plasmid map and multiple cloning sites.

II. **Functional characterization of aromatase expressed --- stable transfection of pTRE-arom vector to MCF-7Tet-off cells.** Since the expression of aromatase gene is under the control of tetracycline response element, functional test requires the cell line that is stably transfected with pTet-Off or pTet-On vector. Our laboratory established MCF-7Tet-Off cell line for other purpose which is suitable for functional characterization of pTRE-arom construct.

We screened 122 MCF-7Tet-Off^{arom} clones. Sixty-one clones expressed aromatase with various levels of activity. Aromatase activity in all positive clones can be inhibited by specific aromatase inhibitor, letrozole, and doxycycline that switches off aromatase gene expression. Our data indicate that pTRE-arom construct can express functional aromatase which is under the tight control of tetracycline.

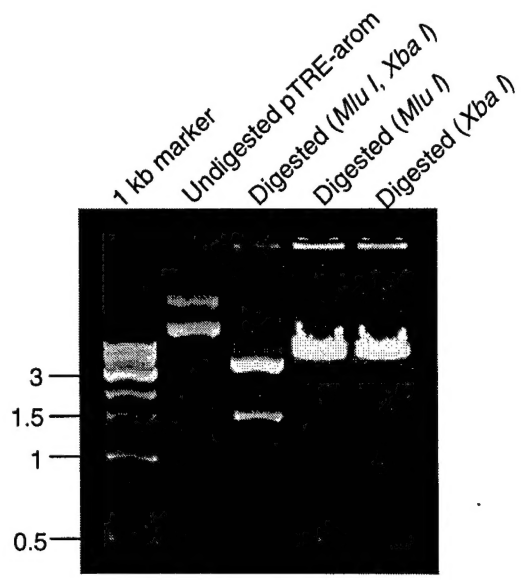

Fig. 2. Restriction enzyme digestion of pTRE-arom vector. Digestion with Mlu I and Xba I resulted in a 1.5 kb insert and a 3.8 kb backbone of pTRE2 vector. Digestion with either Mlu I or Xba I created a linear band with equal molecular size of ~5 kb.

III. **Stable transfection of MCF-10A cells with pTet-Off vector.** In the meantime of verification of pTRE-arom construct, we performed stable transfection of pTet-Off vector into MCF-10A cells to establish a cell line for tetracycline-inducible expression of aromatase. We screened 45 clones after G418 selection for the expression of tetracycline controlled transactivator (tTA). The method used for screening was transient transfection of pTRE-luc and measurement of luciferase activity. The results were unexpected. All clones displayed high luciferase activities and none of them were inhibited by doxycycline. To rule out technical problems that might lead to misinterpretation of the results, we repeated the experiments under altered conditions including changing plasmid and transfection reagent concentrations for transient transfection, usage of electroporation for stable transfection, and employment of pTet-On vector to reduce basal luciferase activity. Consistent results were obtained. We conclude from these data that MCF-10A cells are not suitable for tetracycline-controlled gene expression. Ackland-Berglund and Leib observed similar phenomenon when they performed transient and stable transfection of pTet-On vector into hamster kidney cells (7). High basal luciferase activity and uncontrollable promoter activity by tetracycline in pTet-Off transfected MCF-10A may reflect variations in uptake, localization and half-life of doxycycline and improper interaction of tTA with tetracycline-response element upstream the CMV promoter.

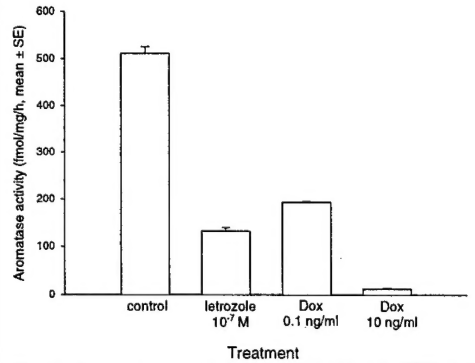

Fig. 3. Aromatase activity of MCF-7Tet-Off^{arom} clone #120 measured by tritiated water release assay. The cells were pretreated with letrozole or doxycycline (Dox) at indicated concentrations for 4 days before the assay.

IV. **Stable transfection of MCF-10A cells with pHβ-aro vector.** As a backup approach, we decided to express aromatase in MCF-10A cells with pHβ-aro vector, a vector being successfully used to generate stable aromatase expressing cells in several different cell lines (8). We screened 66 G418 resistant clones and 23 clones showed aromatase activities. The aromatase activity can be completely inhibited by letrozole, a specific aromatase inhibitor. Fig. 4 shows aromatase activities of six positive clones and the effect of letrozole.

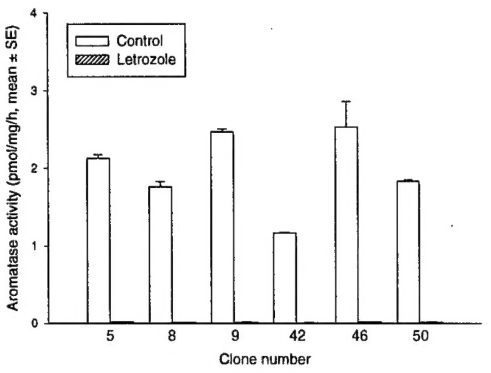

Fig. 4. Aromatase activity of pHβ-aro transfected MCF-10A cells measured by tritiated water release assay. Letrozole (10^{-7} M) was included in two parallel wells during 2 h incubation.

Profound studies of aromatase activity in MCF-10A^{arom} clones selected for future investigation are planned. These include Northern analysis of aromatase mRNA, aromatase activity by product formation analysis, and enzyme kinetics.

KEY RESEARCH ACCOMPLISHMENTS

- Successful construction of pTRE-arom vector that expresses functional aromatase under the control of tetracycline in MCF-7 breast cancer cells.
- Demonstration that benign breast epithelial cell line, MCF-10A, is **NOT** suitable for tetracycline-controlled gene expression.
- Successful establishment of stable MCF-10A^{arom} line that expresses functional aromatase.

REPORTABLE OUTCOMES

- Abstract entitled "**TETRACYCLINE-CONTROLLED EXPRESSION OF AROMATASE**" submitted to *Era of Hope Department of Defense Breast Cancer Research Program Meeting,* 2002.
- Promoted to Associated Professor of Research in July, 2002

CONCLUSIONS

We have fulfilled the tasks stated in Specific Aim 1 as scheduled. We initially constructed an aromatase expressing vector under the control of tetracycline-response element, pTRE-arom. This construct was later demonstrated to express functional aromatase when stably transfected into existing MCF-7 cells that express tetracycline-regulatory transactivator (tTA). Doxycycline, a tetracycline derivative, efficiently switches off aromatase expression. We encountered difficulty in establishing MCF-10A stable line that expresses aromatase under the control of tetracycline. Although we did not obtain proposed stable MCF-10A line that expresses aromatase under the control of tetracycline, our results at least provide other scientists with the information that MCF-10A cells are not suitable for tetracycline-controlled gene expression. We finally used alternative approach to establish a MCF-10A cell line stably expressing aromatase. This cell line will enable us to continue the studies proposed in this project.

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APPENDICES

- **Abstract**

TETRACYCLINE-CONTROLLED EXPRESSION OF AROMATASE

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The relevance of estrogen to established breast cancer is well documented. Substantial correlative data suggest that estrogens cause breast cancer in women. The commonly held mechanism whereby estrogen causes cancer is that estrogen increases the rate of cell division and spontaneous replication errors while reducing time for DNA repair. However, a body of emerging data suggests another mechanism whereby estrogen is metabolized to genotoxic products that directly initiate mutations.

Conversion of androgen substrates to estrogens is catalyzed by aromatase, the rate-limiting enzyme in the estrogen synthesis pathway. While the circulating estrogen concentrations are low in postmenopausal women, estrogen levels in cancerous breast tissues are comparable to those found in breast cancers of premenopausal women. Our prior studies demonstrated that in situ aromatization in the breast play a critical role in determination of tissue estradiol concentration and tumor growth. We hypothesized that overexpression of aromatase in the breast causes breast cancer via metabolite-mediated genotoxicity and estrogen receptor-mediated cell proliferation.

To test this hypothesis, we plan to establish a benign breast epithelial cell line stably expressing aromatase and to determine carcinogenic metabolites and tumorigenesis of the cells. The CLONTECH Tet-Off Gene expression system is used as an approach to express aromatase. Human aromatase cDNA was amplified by PCR, purified, and ligated to pTRE vector. The resultant vector, pTRE-Arom was verified by sequence analysis. Existing MCF-7TetOff cells were stably transfected with pTRE-Arom and selected with G418. Of 86 G418-resistant clones, 42 showed aromatase activities of wide range by tritiated water release assay. Increased conversion of [3H]androstenedione can be inhibited by letrozole, a specific aromatase inhibitor. Treatment with doxycycline (100 ng/ml) for 3-4 days significantly reduced the conversion of [3H]androstenedione suggesting that aromatase expression was turned off. These data indicate that pTRE-Arom construct expresses functional aromatase protein under the control of tetracycline and is ready for controlled expression of aromatase in benign breast epithelial cells.

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